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Manuscript title: Role of HIF-1α and CASPASE-3 in cystogenesis of odontogenic cysts and tumors.

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Abstract

Objectives: Odontogenic cysts and tumors are the most relevant lesions that affect the gnathic bones. These lesions have in common the formation of cystic areas and this common feature may suggest involvement of similar mechanisms. The hypoxia inducible factor 1 alpha (HIF-1α), a responsive protein to hypoxia and caspase-3, an irreversible apoptosis marker, may contribute to cyst formation. Thus, this study aimed to investigate the immunoexpression of these proteins in odontogenic cysts and tumors.

Material and methods: Twenty cases of ameloblastoma, keratocystic odontogenic tumor (KOT) (n=20), radicular cyst (RC) (n=18), dentigerous cyst (DC) (n=11), calcifying cystic odontogenic tumor (n=8) and dental follicle (DF) (n=10) were used to investigate HIF-1α and caspase-3 expression in sequential serial cuts by immunohistochemistry.

Results: HIF-1α was overexpressed in RC, DC and ameloblastoma when compared with DF. The basal and sometimes the lower suprabasal layer showed no or very low expression in DC, KOT and ameloblastoma, the last also showing strong expression in solid epithelial areas and initial cystic formation regions. Caspase-3 was found to be overexpressed in all lesions, with the highest expression in odontogenic cysts compared to tumors. HIF-1α and caspase-3 were localized in similar areas of the same lesions, especially in the epithelium surrounding cystic formations.

Conclusions: This study showed distinct immunoexpression of HIF-1α and caspase-3 in odontogenic cyst and tumors, with higher expression observed in odontogenic cysts.

Clinical relevance: These finding suggesting a possible correlation between hypoxia, apoptosis and cystogenesis, leading to understand the mechanisms responsible to cystic formation in odontogenic lesions.

Keywords: Odontogenic tumors; Odontogenic cyst; HIF-1α; Caspase-3; Cystogenesis

INTRODUCTION

Odontogenic cysts and tumors are lesions that develop exclusively in or around gnathic bones [1, 2]. They are characterized by the presence of a lining epithelium of odontogenic origin and a cystic area containing liquid and/or a fluid material is often observed [1, 2]. Due to their high frequency and/or local aggressiveness, ameloblastoma and keratocystic odontogenic tumor (KOT) represent the most clinical relevant odontogenic tumors, and radicular cyst (RC) and dentigerous cyst (DC) the most important odontogenic cysts. Some odontogenic tumors, such as ameloblastoma, may have cystic and solid areas [2-5]. Little is known regarding the pathogenesis of cyst formation but it is supposed to involve proliferation and death of cells of the lining epithelium [6-8].

There are a few proposed theories about the development cystic areas in odontogenic lesions [9, 10], but majority of them lack of scientific evidence. The strongest and most accepted theory suggests that the development of odontogenic cysts as a result of an increased epithelial proliferation creating large epithelial island, leading to deficient nutricional supply in central areas. Thus, these centrally located cells undergo to necrosis initiating the cystic cavity and also exposing the hyperosmolar intracellular contents, attracting liquid from the surrounding tissues, contributing to cyst growth [10-12].

Odontogenic cysts and tumors are known for a high proliferation rate of the epithelium parenchyma [8, 13-15]. This cellular proliferation will result in an agglomerate of epithelial cells and formation of cellular islands [14, 15]. Because epithelial cells have no direct blood supply, all nutrients required for their metabolism are acquired from the surround connective tissue [16]. This proliferation and cellular aggregation possibly limits the oxygen (O_2) diffusion to the cells in the center of the island, leading to apoptosis and inducing cyst formation [12, 15].

Low oxygen (O_2) concentrations ($O_2 \leq 2\%$) [17] are known to create a condition called hypoxia, which is responsible for triggering several cellular events [18]. Different proteins and transcription factors have been associated to hypoxic conditions, which has in the hypoxia inducible factor 1 alpha (HIF-1α) [19].

Hypoxia increases HIF-1α levels by inhibiting its degradation [20], which induces many different types of cellular responses that include cell proliferation, angiogenesis and apoptosis [21]. Proliferation of the epithelium and apoptosis are fundamental events in cystogenesis [12,22] and have been related to the development of odontogenic cysts and tumors [15, 23]. Hypoxia has already been associated with cyst formation in ameloblastoma [24].

Apoptosis has important roles in a variety of physiological and pathological phenomena [25]. Apoptosis is a well-regulated process and occurs because of the activation of members of the cysteine-aspartic proteases (caspase) family [26]. Activated caspase-3 is considered as an apoptosis marker, due to its critical and irreversible role in the mechanism of controlled cell death [27].

It is believed that cyst formation can be a result of an imbalance between cell proliferation and cell death [28]. Further evidences suggest the modulation of apoptosis by HIF-1α in pathologic and physiologic conditions [28-30]. During hypoxia and consequent overexpression of HIF-1α, the cell can get into apoptotic cell death after initiating a caspase-dependent cascade of events [31].

Many events can occur and influence the microenvironment that surrounds odontogenic lesions. Thus, this study aimed to evaluate HIF-1α and caspase-3 immunoexpression in ameloblastoma, KOT, calcifying cystic odontogenic tumor (CCOT), RC, and DC in order to identify the expression of these proteins and their possible association and participation in the formation of cystic areas of these lesions.

MATERIALS AND METHODS

Samples

Tissue microarrays (TMA) containing 20 ameloblastoma cores (Biomax Inc., Rockville, MD, USA), and tissue sections of odontogenic cysts and tumors were used in this study. Cases of KOT (20), RC (18), DC (11), CCOT (8) with 5 μm thickness completed our sample. Additionally, ten cases of dental follicle (DF) were included as a non-pathological control of normal dental tissue exhibiting neither cystic formation nor neoplastic changes [2]. Samples groups were classified in odontogenic tumors (ameloblastoma, KOT and CCOT) or odontogenic cysts (RC and DC). KOT, RC, DC, CCOT and DF sections were obtained in consecutive cut series from the archives of the department of Oral Pathology of the School of Dentistry of the University Center of Pará (CESUPA, Belém-PA, Brazil). Cystic areas were selected in both odontogenic cysts and tumors. Diagnosis of each lesion was confirmed by a pathologist before experimental procedures. This study was approved by the Human Research Ethics Committee of the Institute of Health Sciences of the Federal University of Pará (CAAE: 36572414.7.0000.0018, nr 877.322/2014).

Immunohistochemistry

Histological sections, obtained from the KOT, RC, DC, CCOT and DF samples, were mounted on glass slides treated with 3-Aminopropyl triethoxysilane (Sigma Chemical Corp, St. Louis, MO, USA). TMA cores of ameloblastoma and samples sections were deparaffinized in xylene and hydrated using decreasing concentrations of ethanol. The slides were immersed in a 20% H_2O_2/methanol (1:1) solution for 20 minutes to inhibit endogenous peroxidase activity. Antigen retrieval was performed for 30 seconds in citrate buffer (pH 6.0) using a Pascal chamber (Dako, Carpinteria, CA, USA). Non-specific antibody binding was blocked using 1% bovine serum albumin (BSA, Sigma Chemical Corp.) in a phosphate-buffered saline solution (PBS) for 1 hour.

The slides were incubated with primary antibodies anti-HIF-1α (1:25, clone H1α67, Merck Millipore, Darmstadt, Germany) and anti-caspase-3 (1:100, clone CPP32, Diagnostic BioSystems, Pleasanton CA, USA), which reacts against with the active form of this protein, for 1 hour and followed by a 30-minute incubation with EnVision Plus detection system (Dako®). Diaminobenzidine (Dako®) was used as the chromogen. Sections were counterstained with Mayer's hematoxylin (Sigma Chemical Corp.) and mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA). As a negative control, the primary antibodies were replaced by non-immune serum. Squamous cell carcinoma samples were used as positive control for the immunohistochemical reaction.

Immunostaining evaluation

The immunostaining evaluation was performed by analyzing the percentage of HIF-1α and caspase-3 stained area in the epithelial layer in all samples. Brightfield images of five randomly selected

areas were acquired for each sample using AxioScope microscope equipped with an AxioCamHRc CCD color camera (Carl Zeiss, Oberkochen, Germany), with a 40× objective. For this step, the sites selected for quantification were from cystic areas, from both odontogenic cists and tumors. Diaminobenzidine stained areas were separated and segmented using the "deconvolution color plug-in" (Gabriel Landini, http://www.dentistry.bham.ac.uk/landinig/software/software.html) from ImageJ software (public domain software, NIMH, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/). After image segmentation, the fraction of stained area was measured. Differences between ameloblastoma, KOT, RC, DC, CCOT and DF immunoexpression were then analyzed. The samples were evaluated by one examiner in a blinded study towards diagnosis. The evaluation of expression of HIF-1a and caspase 3 in odontogenic cysts and tumors was conducted descriptively.

Statistical analysis

The data was analyzed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Following normality analysis, a two-way ANOVA followed by Tukey's multiple comparison test was used to evaluate the differences in HIF-1α and caspase-3 stained areas between samples and control.

RESULTS

HIF-1α and caspase-3 were expressed in odontogenic cysts and tumors

All samples of odontogenic cysts and tumors analyzed in this study expressed HIF-1α and activated caspase-3 (Figures 1-5). Differences among these lesions were evaluated in consideration of staining intensity, cellular immunolocalization and epithelial layers distribution. Subsequent cuts from the same area were analyzed in parallel for HIF-1α and caspase-3 immunoexpression.

In ameloblastoma TMA cores, HIF-1α expression (Figures 1A, 1C, 1E and 1G) was observed as intense nuclear (Figure 1C, arrowhead) and cytoplasmic staining (Figure 1C, arrow) in the epithelial layer near and/or in close contact with the cystic lumen (Figures 1A and 1C, asterisk). On the other hand, the cells of the basal and lower suprabasal layer, showed a week cytoplasmic staining. There was an intense nuclear and cytoplasmic staining in epithelial islands, where cells presenting large vacuoles areas could be observed (Figure 1E, arrow), and also in cells surrounding initial cystic formation (Figure 1E, asterisk). The cells located in the solid epithelial proliferations of tumor parenchyma displayed intense nuclear and cytoplasmic (Figure 1G, arrow) HIF-1α staining.

Caspase-3 analysis (Figures 1B, 1D, 1F e 1H) was carried out using similar regions of the ameloblastoma TMA cores compared to those of HIF-1α. Caspase-3 immunostaining predominated in the cytoplasm of cells near to cystic areas (Figures 1B and 1D, asterisk) of the epithelial islands and nuclear and cytoplasmic staining in selected cells of the basal layer (Figure 1D, arrow). In tumor parenchyma, areas of cyst formation (Figures 1F, asterisk) and solid areas of epithelial islands showed almost an exclusively cytoplasmic staining for caspase-3 (Figures 1F and 1H, arrow).

All KOT samples showed colocalized HIF-1α and caspase-3 staining (Figure 2). HIF-1α immunoexpression was observed in both cellular compartments, but predominantly located in the

parabasal layer near to the cystic cavity (Figures 2A and C, asterisk) in cytoplasm (Figure 2C, arrow) and nucleus (Figure 2C, arrowhead). No HIF-1α staining was observed in the basal and lower suprabasal layer. On the other hand, caspase-3 (Figures 2B and 2D) showed cytoplasmic (Figure 2D, arrow) and nuclear (Figure 2D, arrowhead) expression in selected neoplastic cells of all epithelial layers. Expression of caspase-3 appeared to be less intense in the basal layer.

In RC, HIF-1α (Figures 3A and 3C) and caspase-3 (Figures 3B and 3D) were similarly observed in the cell nucleus (Figures 3C and 3D, arrowhead) and cytoplasm (Figures 3C and 3D, arrow) of cystic epithelium. In cystic stroma, inflammatory cells were positively stained for both proteins (Figures 3E and 3F, arrow).

In DC samples, HIF-1α (Figures 4A and 4C) showed a similar expression to those observed in KOT samples, with cytoplasmic (Figure 4C, arrow) and nuclear staining (Figures 4C, arrowhead) of the suprabasal cells and no staining of the basal layer. However, caspase-3 staining showed a predominantly cytoplasmic immunolocalization especially in the suprabasal layer (Figure 4D), with some basal cells showing both, nuclear and cytoplasmic staining (Figure 4D, arrow).

HIF-1α and caspase-3 staining showed a similar pattern in CCOT samples. The expression of these proteins was localized in the cytoplasm and distributed similarly in all epithelial layers (Figures 5A and 5B). The DF exhibited weak HIF-1α and moderate caspase-3 staining in the cytoplasm of the epithelial cells (Figures 5C and 5D).

HIF-1α is highly expressed in ameloblastoma, DC, and comparatively higher in RC when compared to odontogenic tumors

HIF-1α expression showed variable expression in all samples (Figure 6A). The highest expression was observed in RC (Mean= 58.11; SD=11.61), which was statistically significant when compared to odontogenic tumors (ameloblastoma p=0.026, Mean=44.69, SD=13.87; KOT p<0.0001,; CCOT p<0.0001). Stained area of KOT (Mean=36,20, SD=15.17) and CCOT (Mean=17.60, SD=12.77) was not statistically different from control (Mean=23.07, SD=6.35), and HIF-1α expression in CCOT was lower when compared with all other lesions (ameloblastoma p=0.0004; KOT p=0.0368; DC p=0.0005, Mean=46,25, SD=13.50; and RC p<0.0001). Although there was not difference between HIF-1α immunostaining between control and CCOT, CCOT expression was lower than all the other studied lesions.

Caspase-3 is highly expressed in odontogenic cysts compared to odontogenic tumors.

Results of caspase-3 immunostaining quantification demonstrated that odontogenic cysts showed higher percentage of stained areas than odontogenic tumors (Figure 6B). DC (Mean=65,57, SD=13.86) showed the highest expression of caspase-3, followed by RC (Mean=60.23; SD=7.58), ameloblastoma (Mean=50.16, SD=6.63) and KOT (Mean=49.44, SD=8.23). Comparison between odontogenic cysts and tumors revealed higher expression in DC when compared to ameloblastoma (p=0.0005), KOT (p=0.0002)

and CCOT ($p<0.0001$, Mean=43.32, SD=13.14) and also higher expression in RC against the same ameloblastoma ($p=0.0175$), KOT ($p=0.0086$) and CCOT ($p<0.0016$). No differences were observed when compared odontogenic cysts or tumors with each other. Caspase-3 expression was significantly higher in all studied samples ($p<0.0001$) when compared to the non-pathological control of DF (Mean=13.82, SD=10.53).

DISCUSSION

Although the pathogenesis of odontogenic lesions may differ in developmental, inflammatory or neoplastic lesions, they can present a common feature that is cystic formation. The reasons why it happens is poorly understood, which arises the question why lesions of different pathogenesis display similar pathological features. It is considered that many of these processes start with proliferation of the odontogenic epithelium [9, 10]. According these cells grow, the central cells start to have more restricted access to oxygen, leading them to hypoxia. In this study was observed a high expression of the hypoxia-related protein HIF-1α in odontogenic cysts and ameloblastoma and also high immunoexpression of caspase-3 in all odontogenic cysts and tumors.

HIF-1α is the main cellular protein in response to hypoxic conditions [21]. Although HIF-1α is normally produced, it is quickly degraded in normoxic condition [19]. HIF-1α accumulation is only possible at low oxygen concentration levels [19]. Thus, in this context HIF-1α can be considered a hypoxia marker [32]. In this study, odontogenic cysts showed the highest immunoexpression of HIF-1α, suggesting that these lesions undergo to restricted oxygen supply. Ameloblastoma samples also showed increased expression of HIF-1α in solid areas of epithelial islands, supporting the idea of localized intratumoral hypoxia [24].

KOT, DC and cystic areas of ameloblastoma showed an interesting pattern of HIF-1α immunostaining. The basal and, in some cases, the suprabasal layer showed no HIF-1α immunostaining, while the upper layers close to the cystic area and more distant from the connective tissue were positively stained. It strongly suggests a reduced oxygen supply when the cells distantiate from the basal layer, which normally occurs as result of proliferation. This situation leads to stabilization of HIF-1α activating its response.

Upon activation, HIF-1α can modulate many cellular responses, including inflammation and apoptosis [32]. This study showed that odontogenic cysts and ameloblastoma have a higher expression of HIF-1α when compared to DF. It is more evident in RC, which showed an extensive inflammatory infiltrate in the stroma [33].

We then hypothesize that with proliferation of the epithelial cells from basal layer, the cells of the upper layers experience limited O_2 supply, causing a hypoxia-related response overexpressing HIF-1α. Under certain conditions, the protective mechanism to hypoxia that initiates HIF-1α response is not enough to maintain cell viability, thus triggering a more extreme response that leads to apoptosis throughout activation of a caspase cascade, analyzed here by the expression of the irreversible apoptosis marker activated caspase-3. Caspase-3 and HIF-1α were observed in co-localization in this study,

suggesting that these processes can be associated and together may play a role in formation of cystic areas.

HIF-1α is known to induce cellular apoptosis [30, 34, 35] and also a key molecule [36, 37] that can overcome stabilization mediated by the p53 suppressor gene [38]. HIF-1α can promote the release of cytochrome C to the mitochondria and subsequent activation of caspase-3 [31, 39, 40]. This study pointed out that HIF-1α expression was higher in odontogenic cysts and ameloblastoma. Overexpression of p53 suppressor gene is a common found in ameloblastoma [41], which also showed p53 gene mutations and allelic loss [42].

Previous studies showed that HIF-1α knockout suppresses the apoptosis induced by p53 [30] and significantly reduces cyst formation [34, 43]. There is strong evidence of a correlation between HIF-1α accumulation and caspase-3 activation [44]. In our samples, HIF-1α and caspase-3 were found to be expressed in the same areas of all lesions, which support the association between those two molecules. An interesting immunolocalization pattern with higher expression of both proteins when the cells were located more distant from the basal layer was observed in cases of ameloblastoma, KOT and DC.

Caspase-3 staining in RC and DC was mostly nuclear and cytoplasmic, while in odontogenic tumors was predominantly cytoplasmic. DNA fragmentation is a major step in eliminating the genome of apoptotic cells and subsequent cell death [45]. Hence, intense nuclear staining indicates the beginning of apoptosis in odontogenic cysts. Additionally, apoptosis can also be a protection against oxidative stress or other forms of DNA damage and thereby reduce the risk of neoplastic transformation [46].

Ameloblastoma showed a strong nuclear and cytoplasmic staining of HIF-1α and caspase-3 in specific solid areas of epithelial islands, which are known to present difficulties in O_2 and nutrient diffusion [12, 22], and also in surrounding areas of initial cyst formation. This could resemble the ideal environment were hypoxia is established [47] and apoptosis will take place to form future cystic regions. Some cells in epithelial areas of ameloblastoma presented large vacuoles areas, which is a classical sign of cell death [48, 49]. Low expression of CCOT was observed for both proteins compared to the other lesions. CCOT is a non-aggressive odontogenic neoplasm that shows an indolent behavior and low recurrence rates, which has been widely used as a control group for studying odontogenic tumors [50-54].

Although this study can not prove that these events are occurring in consequence of the same cause (hypoxia), the results observed here support this hypothesis and suggest that there might have an association between hypoxia and apoptosis, especially in cases of odontogenic cysts and ameloblastoma. HIF-1α and caspase-3 immunoexpression and immunolocalization patterns suggest an important association between hypoxia, apoptosis and cyst formation. Therefore, HIF-1α and caspase-3 expression labelling in odontogenic cysts and tumors may be important to explain which cellular components are related to the behavior of these lesions. Additionally, these molecules can be used as biological markers in the future, serving as instruments for better diagnosis and treatment planning.

Compliance with Ethical Standards

Conflict of Interest: The author Natacha Malu Miranda da Costa declare that she has no conflict of interest. The author Adriane Sousa de Siqueira declare that she has no conflict of interest. The author Andre Luis Ribeiro Ribeiro declare that he has no conflict of interest. The author Maria Sueli da Silva Kataoka declare that she has no conflict of interest. The author Sérgio Melo de Alves-Junior declare that he has no conflict of interest. The author Andrew M. Smith declare that he has no conflict of interest. The author João de Jesus Viana Pinheiro declare that he has no conflict of interest.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent: For this type of study, formal consent is not required.

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FIGURE LEGENDS

Figure 1: HIF-1α and caspase-3 immunostaining in ameloblastoma. HIF-1α staining (A C, E, G) was predominant in the nucleus (C, arrowhead) and cytoplasm (C, arrow) in epithelial layer near cystic lumen (A, C, asterisk). Intense nuclear and cytoplasmic staining in epithelial islands (E and G,

arrow), and in cells surrounding initial cystic formation was observed (E, asterisk). Caspase-3 immunostaining (B,D,F and H) is predominant in the cytoplasm of cells near cystic areas of epithelial islands (B and D, asterisk). Staining is also detected in selected cells of basal layer (D, arrow). In tumor parenchyma, areas of cyst formation (F, asterisk) and solid areas of epithelial islands showed almost an exclusively cytoplasmic staining for caspase-3 (F and H, arrow). Scale bar: 50 µm and 20 µm.

Figure 2: HIF-1α and caspase-3 immunostaining in KOT. HIF-1α immunoexpression was predominantly located in the parabasal layer near to the cystic cavity (A and C, asterisk) in cytoplasm (C, arrow) and nucleus (C, arrowhead). Caspase-3 (B and D) showed cytoplasmic (D, arrow) and nuclear (D, arrowhead) expression in selected neoplastic cells of all epithelial layers. Expression of caspase-3 appeared to be less intense in the basal layer. Immunoperoxidase. Scale bars: 50 µm and 20 µm.

Figure 3: HIF-1α and caspase-3 immunostaining in RC. Expression of HIF-1α (A and C) and caspase-3 (B and D) were similarly observed in the cell nucleus (C and D, arrowhead) and cytoplasm (C and D, arrow) of cystic epithelium. In cystic stroma, inflammatory cells were positively stained for both proteins (E and F, arrow). Immunoperoxidase. Scale bars: 50 µm and 20 µm.

Figure 4: HIF-1α and caspase-3 immunostaining in DC. HIF-1α (A and C) showed a cytoplasmic (C, arrow) and nuclear staining (C, arrowhead) of the suprabasal cells and no staining of the basal layer. Caspase-3 staining showed a predominantly cytoplasmic immunolocalization especially in the suprabasal layer (D). Some basal cells showing both, nuclear and cytoplasmic staining (D, arrow). Immunoperoxidase. Scale bars: 50 µm and 20 µm.

Figure 5: HIF-1α and caspase-3 immunolabeling in CCOT and DF. HIF-1α and caspase-3 staining showed a similar pattern in CCOT samples (A and B). The DF exhibited weak HIF-1α and moderate caspase-3 staining in the cytoplasm of the epithelial cells (C and D). Immunoperoxidase. Scale bars: 50 µm and 20 µm.

Figure 6. Comparison of HIF-1α immunostaining between ameloblastoma, KOT, CCOT, RC and DC (A). Comparison of caspase 3 immunostaining between ameloblastoma, KOT, CCOT, RC and DC (B). Dental follicle was used as control. AME-Ameloblastoma; KOT-Keratocystic odontogenic tumor; CCOT-Calcifying cystic odontogenic tumor; RC-Radicular cyst; DC-Dentigerous cyst; DF-Dental follicle. Significance: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Figure 1

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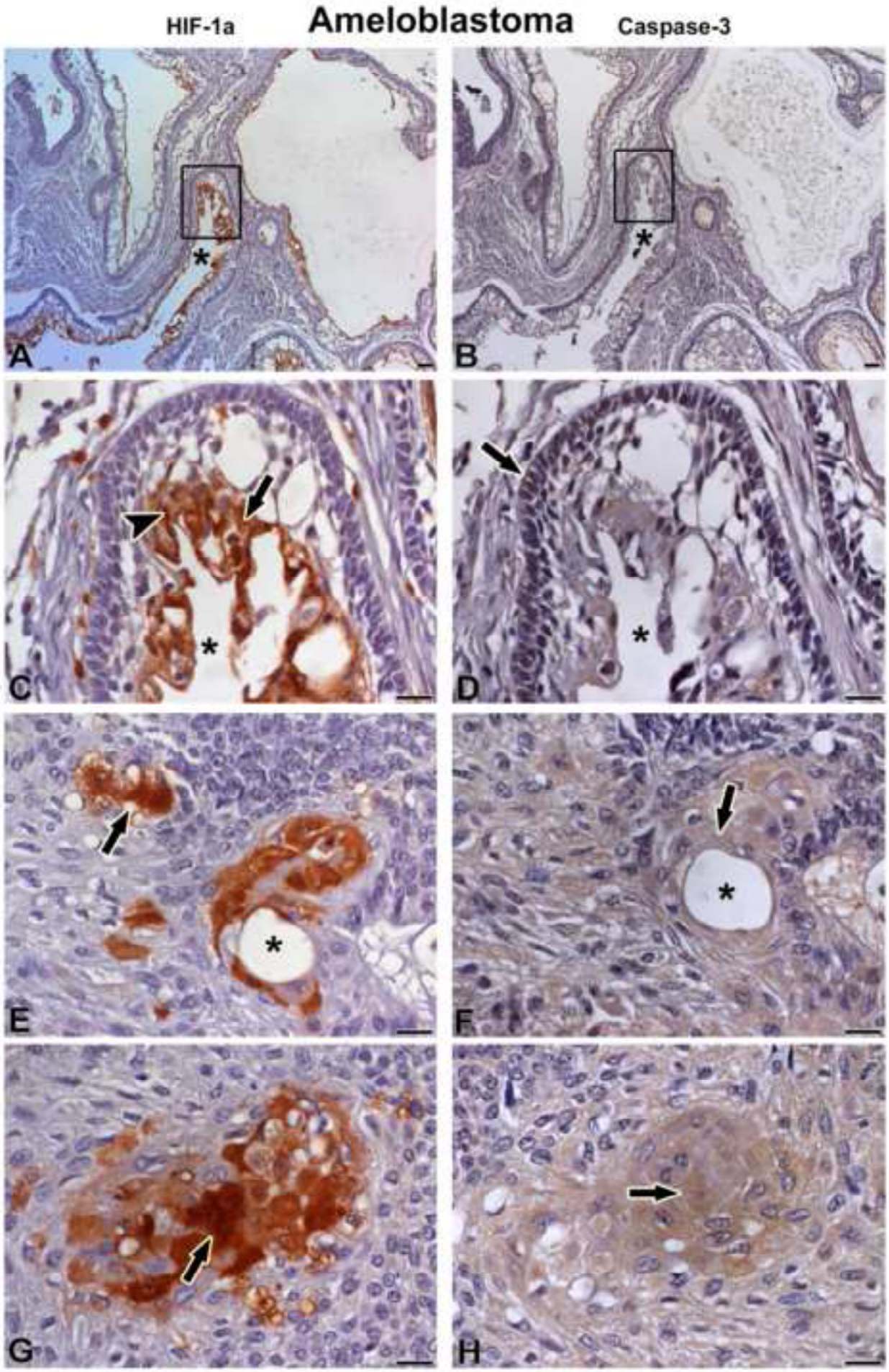

Figure 2

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Figure 2

KOT

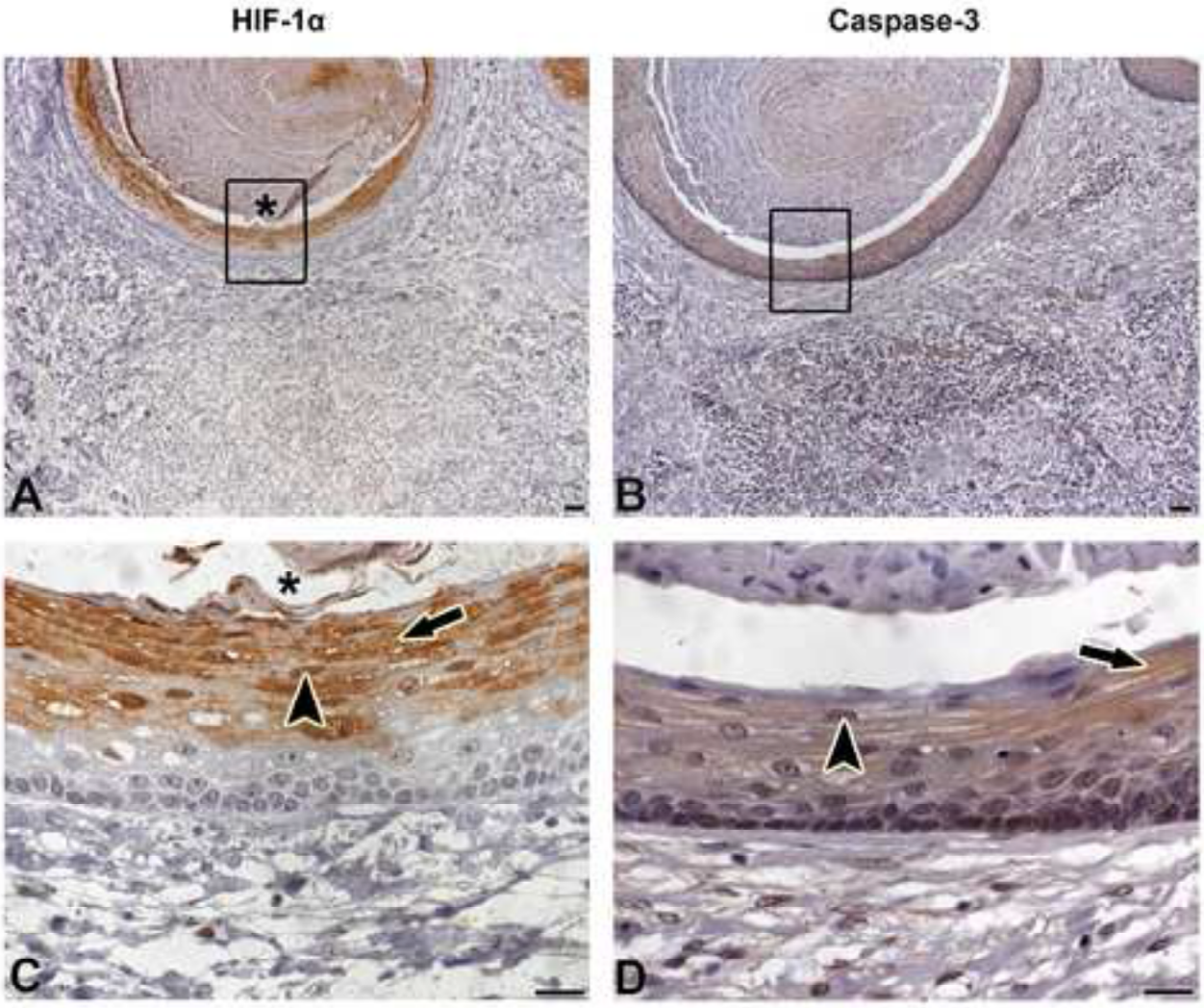

Figure 3

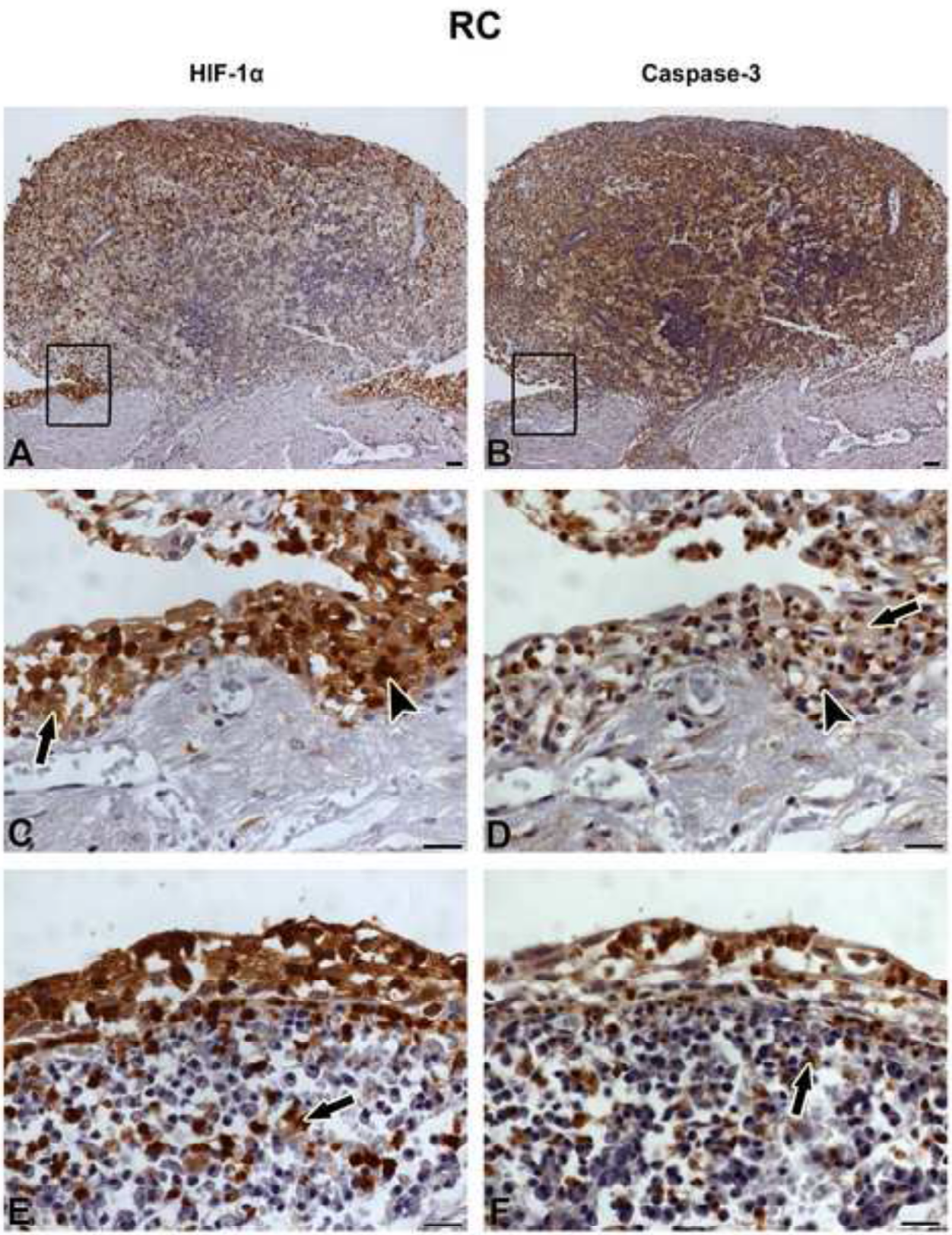

Figure 4

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Figure 4

DC

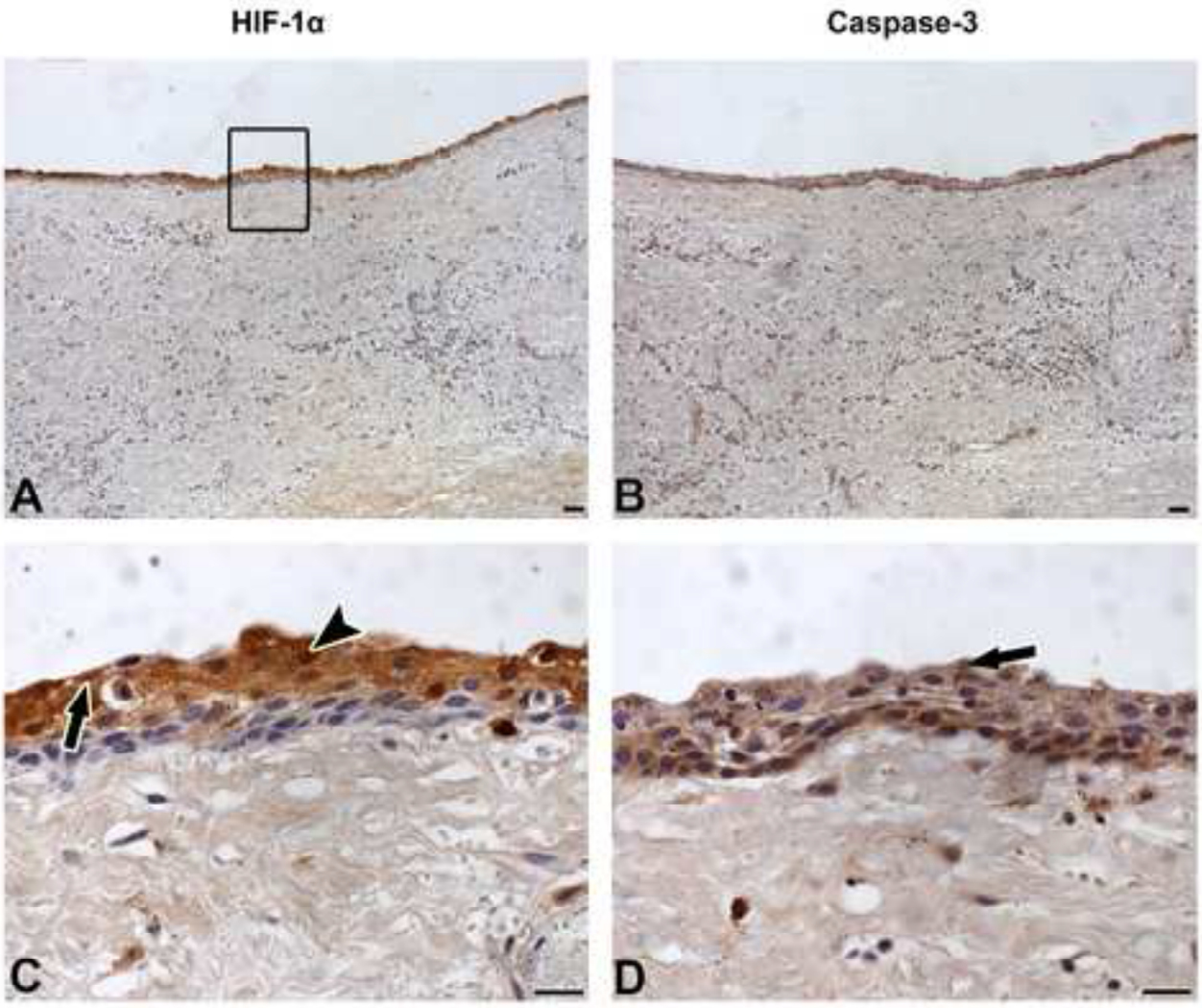


Figure 5

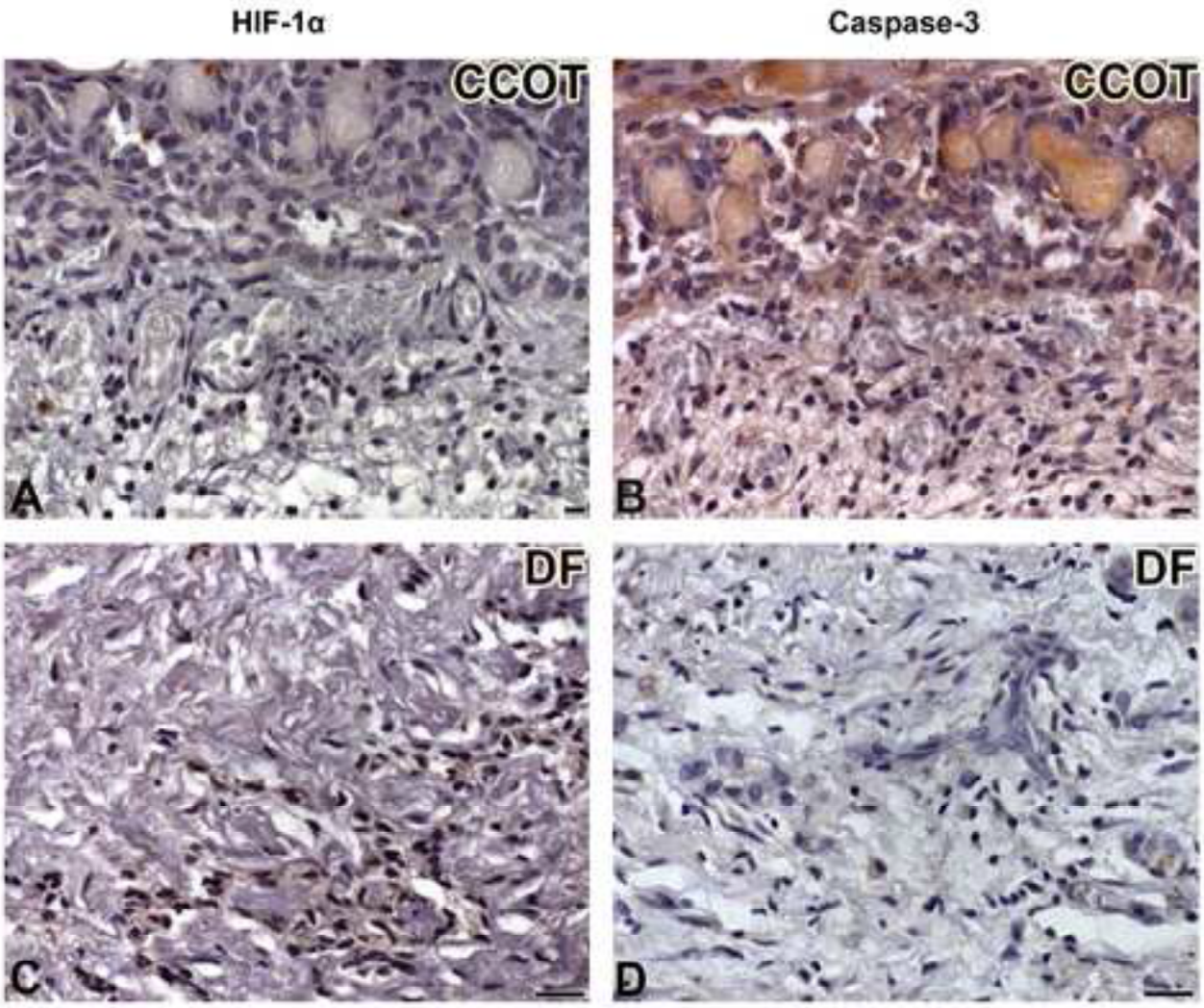
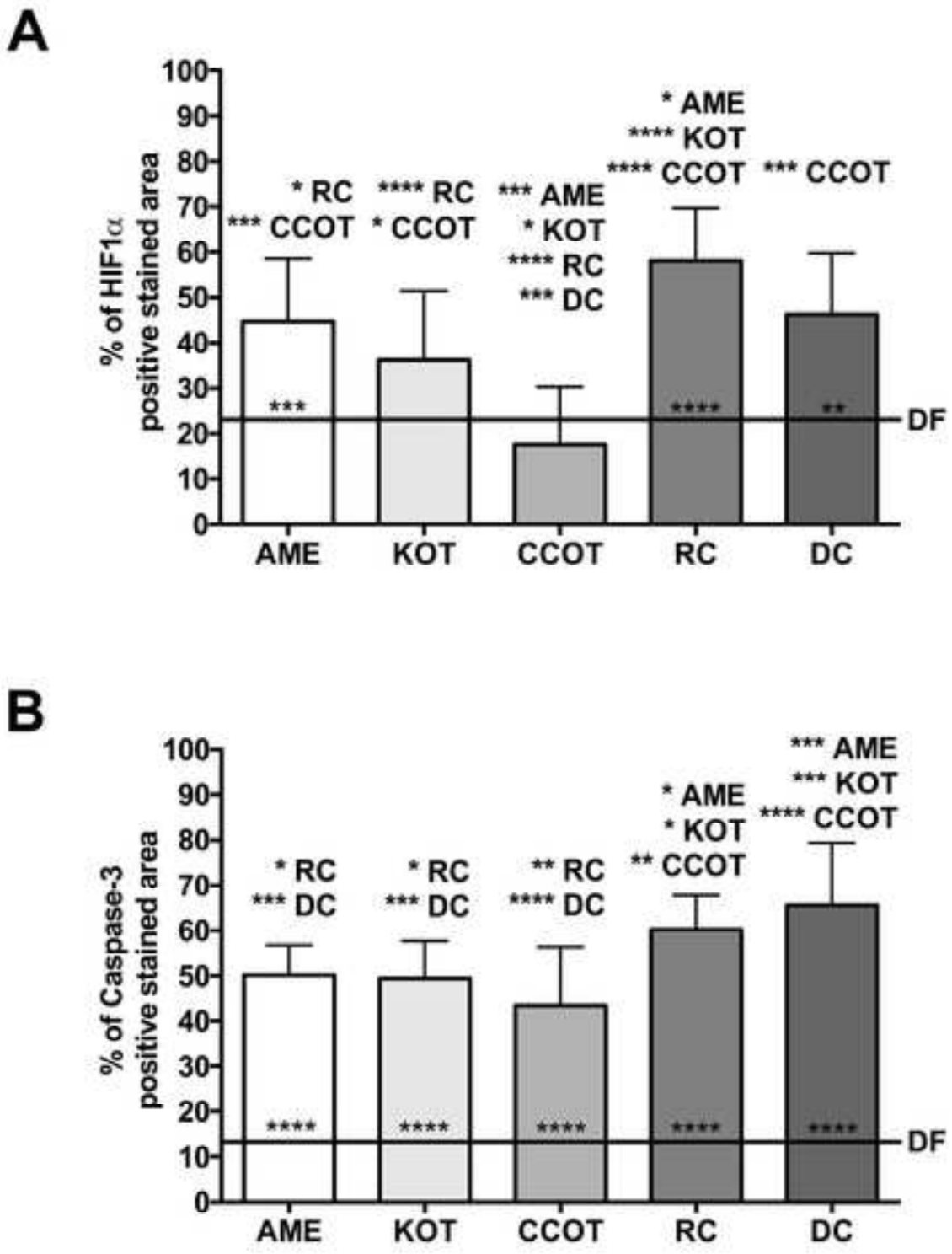


Figure 6



UNIVERSIDADE FEDERAL DO PARÁ
INSTITUTO DE CIÊNCIAS DA SAÚDE
FACULDADE DE ODONTOLOGIA
LABORATÓRIO DE CULTIVO CELULAR

Belém, February 2th, 2016.

Dr Matthias Hanning
Editor
Clinical Oral Investigations
Dr. Hanning,

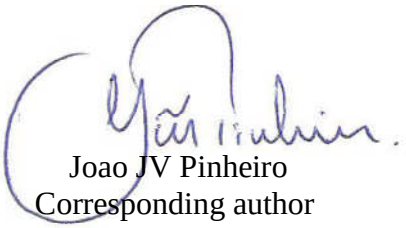
Please find enclosed the revised manuscript CLOI-D-16-01334 entitled "RELATIONSHIP AMONG HIF-1α, CASPASE-3 AND CYSTOGENESIS IN ODONTOGENIC CYSTS AND TUMORS".

We were pleased to see that the reviewer recommended publication, suggesting some revisions of the manuscript. All corrections made are highlighted in the manuscript. We thank the reviewers for the interesting suggestions, which have improved the manuscript.

We have included a point-by-point reply to reviewer's concerns comments below and hope the manuscript will be acceptable for publication in Clinical Oral Investigations.

Hoping to hear from you at your earliest convenience, we thank you for your kind attention

Sincerely,



Joao JV Pinheiro
Corresponding author

Reply to reviewer #1

1. Concern of the reviewer

Very interesting approach to research into cyst Formation. Legend of Figure 1 is much to long, should be shortended.

Our response:

The legend of figure 1 (Figure legends, page 11, last paragraph, page 12 first paragraph) was shortened.

Revised text:

"Figure 1: HIF-1α and caspase-3 immunostaining in ameloblastoma. HIF-1α staining (A C, E, G) was predominant in the nucleus (C, arrowhead) and cytoplasm (C, arrow) in epithelial layer near cystic lumen (A, C, asterisk). Intense nuclear and cytoplasmic staining in epithelial islands (E and G, arrow), and in cells surrounding initial cystic formation was observed (E, asterisk). Caspase-3 immunostaining (B,D,F and H) is predominant in the cytoplasm of cells near cystic areas of epithelial islands (B and D, asterisk). Staining is also detected in selected cells of basal layer (D, arrow). In tumor parenchyma, areas of cyst formation (F, asterisk) and solid areas of epithelial islands showed almost an exclusively cytoplasmic staining for caspase-3 (F and H, arrow). Scale bar: 50 μm and 20 μm."

Reply to reviewer #2

1. Concern of the reviewer

Introduction: Adequate. Though, due the high number of different lesions, the authors are encouraged to differentiate between odontogenic tumors and cysts at this stage of the manuscript.

Our response:

We included a sentence differentiating odontogenic tumors and cysts in the first paragraph of Introduction section (page 3).

Revised text:

"Odontogenic cysts and tumors are lesions that develop exclusively in or around gnathic bones [1, 2]. They are characterized by the presence of a lining epithelium of odontogenic origin and a cystic area containing liquid and/or a fluid material is often observed. [1, 2]. Due to their high frequency and/or local aggressiveness, ameloblastoma and keratocystic odontogenic tumor (KOT) represent the most clinical relevant odontogenic tumors, and radicular cyst (RC) and dentigerous cyst (DC) the most important odontogenic cysts. Some odontogenic tumors, such as ameloblastoma, may have cystic and solid areas. [2-5]".

2. Concern of the reviewer

Materials and methods: Was the diagnosis of the lesions confirmed by a pathologist before further evaluation?

Our response:

The diagnostic of each lesion was confirmed for a pathologist before evaluation. We now explain that issue in Samples section of Materials and methods (page 4, second paragraph).

Revised text:

"Diagnosis of each lesion was confirmed by a pathologist before experimental procedures"

3. Concern of the reviewer

From which site of the lesions the samples were taken?

Our response:

The sites selected for quantification were from cystic areas, both odontogenic cists and tumors. Materials and methods, Samples (page 5, first paragraph).

Revised text:

MATERIALS AND METHODS

Samples

Tissue microarrays (TMA) containing 20 ameloblastoma cores (Biomax Inc., Rockville, MD, USA), and tissue sections of odontogenic cysts and tumors were used in this study. Cases of KOT (20), RC (18), DC (11), CCOT (8) with 5 µm thickness completed our sample. Additionally, ten cases of dental follicle (DF) were included as a non-pathological control of normal dental tissue exhibiting neither cystic formation nor neoplastic changes [2]. Samples groups were classified in odontogenic tumors (ameloblastoma, KOT and CCOT) or odontogenic cysts (RC and DC). KOT, RC, DC, CCOT and DF sections were obtained in consecutive cut series from the archives of the department of Oral Pathology of the School of Dentistry of the University Center of Pará (CESUPA, Belém-PA, Brazil). Cystic areas were selected in both odontogenic cysts and tumors. Diagnosis of each lesion was confirmed by a pathologist before experimental procedures.

4. Concern of the reviewer

How many examiners did the evaluation procedures? Were they blinded towards the diagnosis? Did the authors calibrate their parameters such as staining intensity, cellular immunolocalization as well as epithelial layer distribution?

Our response:

The samples were evaluated by one examiner in a blinded study towards diagnosis. We included this information in Immunostaining evaluation section of Materials and methods (page 5, first paragraph).

Revised text: The samples were evaluated by for one examiner in a blinded study towards diagnosis.

5. Concern of the reviewer

For evaluation of expression on cysts and tumors, please add that this was conducted descriptively.

Our response:

We included this information in Immunostaining evaluation section of Material and Methods (page 5, first paragraph).

Revised text:

"The evaluation of expression of HIF-1a and caspase 3 in odontogenic cysts and tumors was conducted descriptively."

6. Concern of the reviewer

Results: For quantification of expression, please add mean/median values as well as standard deviations to the manuscript.

Our response:

The mean and standard deviations were included into manuscript. The Results section, (page 6, 6th and 7th paragraphs, and page 7, first paragraph).

Revised text:

HIF-1α is highly expressed in ameloblastoma, DC, and comparatively higher in RC when compared to odontogenic tumors

HIF-1α expression showed variable expression in all samples (Figure 6A). The highest expression was observed in RC (Mean= 58.11; SD=11.61), which was statistically significant when compared to odontogenic tumors (ameloblastoma p=0.026, Mean=44.69, SD=13.87; KOT p<0.0001,; CCOT p<0.0001). Stained area of KOT (Mean=36,20, SD=15.17) and CCOT (Mean=17.60, SD=12.77) was not statistically different from control (Mean=23.07, SD=6.35), and HIF-1α expression in CCOT was lower when compared with all other lesions (ameloblastoma p=0.0004; KOT p=0.0368; DC p=0.0005, Mean=46,25, SD=13.50; and RC p<0.0001). Although there was not difference between HIF-1α immunostaining between control and CCOT, CCOT expression was lower than all the other studied lesions.

Caspase-3 is highly expressed in odontogenic cysts compared to odontogenic tumors.

Results of caspase-3 immunostaining quantification demonstrated that odontogenic cysts showed higher percentage of stained areas than odontogenic tumors (Figure 6B). DC (Mean=65,57, SD=13.86) showed the highest expression of caspase-3, followed by RC (Mean=60.23; SD=7.58), ameloblastoma (Mean=50.16, SD=6.63) and KOT (Mean=49.44, SD=8.23). Comparison between odontogenic cysts and tumors revealed higher expression in DC when compared to ameloblastoma (p=0.0005), KOT (p=0.0002) and CCOT (p<0.0001, Mean=43.32, SD=13.14) and also higher expression in RC against the same ameloblastoma (p=0.0175), KOT (p=0.0086) and CCOT (p<0.0016). No differences were observed when compared odontogenic cysts or tumors with each other. Caspase-3 expression was significantly higher in all studied samples (p<0.0001) when compared to the non-pathological control of DF (Mean=13.82, SD=10.53).

7. Concern of the reviewer

Did the authors try to add mean values from either tumors as well as cysts and compare them to each other?

Our response:

After labeling quantification, we found the mean values for each odontogenic cysts and tumors sample, and compared to each other, as demonstrated on Figure 6. Asterisks in Figure 6 indicate which of these comparisons were significant.

Revised text: Not applicable

8. Concern of the reviewer

Discussion: Adequate. Is there a clinical implication of these findings?

Our response:

We think that there is clinical implications of HIF-1° and caspase-3 expression in odontogenic cysts and tumors. We commented this issue on the last paragraph of the section.

Revised text:

"Therefore, HIF-1α and caspase-3 expression in odontogenic cysts and tumors may be important to explain which cellular components are related to the behavior of these lesions. Additionally, these molecules can be used as biological markers in the future, serving as instruments for better diagnosis and treatment planning."